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1. Chong, M. W. et al., Int. J. Pharmaceutics (Jun 1998) 167(1-2): 25-36

2. Chen, B.-L. et al. J. Pharmaceutical Sciences (1994) 83(12): 1657-1661

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Refolding of soluble leukemia inhibitory factor receptor fusion protein (gp 190 sol DAF) from urea

Houqi Liu,[1] Jean-François Moreau,[2] Norbert Gualde[2] and Jiliang Fu[1]

[1]Medical Molecular Biology Laboratory, Second Military Medical University, Shanghai, 200433, Peoples Republic of China; [2]Laboratoire d'Immunologie, Université de Bordeaux II, Bordeaux, France

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Abstract

The insoluble inclusion bodies of soluble leukemia inhibitory factor receptor fusion protein (gp 190 sol DAF) was solubilized in 8 M urea on the unfolding transitions, and several factors on the aggregate formation were indirectly analyzed for the refolding of gp 190 sol DAF. Results indicate that the refolding yield can be considerably increased at lowering concentration of the unfolding protein, a little soluble protein with the slow refolding appears in the process of the aggregate formation and the concentration of the denaturant must be down to a minimum level for its refolding. (Mol Cell Biochem **169**: 43–50, 1997)

Key words: recombinant protein, refolding, urea, glycerol

Introduction

Leukemia inhibitory factor (LIF) is a glycoprotein growth and differentiation regulator that has pleiotropic activity in several adult and embryonic systems. LIF action is mediated following binding to specific cellular receptors that trigger differentiation-induction, differentiation-suppression, proliferation or activation of hematocyte. LIF is known to bind a cloned gp 130-related receptor component with a molecular weight of approximately 190 kDa (gp 190) [1].

Decay accelerating factor (DAF) belongs to a group of membrane proteins anchored to the cell surface by a glycophospholipid membrane anchor that is covalently attached to the carboxyl terminus of the protein. The last 37 amino acid of membrane DAF, when fused to the carboxyl terminus of a secreted protein, are sufficient to target the fusion protein to the plasma membrane by means of the glycophospholipid anchor [2]. LIF soluble receptor (gp 190 sol containing the signal sequence and extracellular domain) was fused with the carboxy-terminus part of membrane DAF (190 sol DAF) to express the soluble receptor on the cell surface, to observe the fusion protein yield, and analyse LIF receptor functions using anti-soluble LIF receptor antibodies.

The cloning of foreign genes in *Escherichia coli* plasmids and their expression after transformation of cells are common procedures in recombinant DNA-based biotechnology methods. The making of fusion proteins which can be purified efficiently by taking advantage of the specific binding of the ligand moiety to an immobilized matrix, often allows a simple one-step purification process. In the present study, the expression system pGEX-5T was used for the construction of fusion gene with cDNA of 190 sol DAF in *Escherichia coli* (XL1), since such a fusion protein can be efficiently purified by the specific binding of the ligand moiety to glutathione Sepharose 4B by means of the glutathione S-transferase (GST) gene fusion system of pGEX-5T [3]. However, successful purification depends entirely on the natural conformation of the synthesized fusion proteins. In general, most of the expressed proteins accumulate intracellularly as insoluble inclusion bodies [4]. It is necessary that the inclusion bodies are solubilized in detergents, in strong chaotropic agents such as guanidinium-hydrochloride, and in urea, all of which mainly affect hydrogen bonding. These agents destroy the natural conformation of the recombinant proteins. So, refolding of proteins treated with denaturants is very important for the purification of recombinant active proteins.

Address for offprints: H. Liu, Room 306, Building 9, 871 Xiangyin Road, Shanghai, 200433, Peoples Republic of China

The expression of gp 190 sol DAF in XL1 strain has been successful [5]. The fusion protein can be solubilized in 8 M urea. But, it fails to be purified by affinity chromatography using a glutathione-Sepharose-4B column because of the unfolding transitions of the fusion protein.

Aggregation and precipitation of the fusion protein after removing the denaturant are often thought to as the consequences of failure of folding experiments, whereas they are often the cause of failure. So, we report here the observation of the influence of several factors on the aggregate formation to analyse indirectly the refolding of gp 190 sol DAF.

Materials and methods

Construction of pGEX-5T 190 sol DAF

The vector pGEX-5T (provided by Dr. W. Northmann, Germany) is a plasmid measuring 4999 bp in length ad directs high-level expression of a fusion protein with the histidine-hexapeptide and GST at its N-terminus and the recombinant protein at its C-terminus.

The 2.7 kbp cDNA coding for the soluble LIF receptor with DAF was isolated from pBSKS gp 190 full length. First, 190 sol in pBSKS was from the subcloning of the 190 full length digested by Pst1 (in FN domain) and Xba1 (in the place stop) as a vector ad 295 bp (FN domain + Xba1 site, PCR construction) as an insert; Then, pBSKS 190 sol was cut with Xba1 and Not1 and ligated to a fragment of 135 bp obtained by PCR from the part of the human DAF (Xba1 DAF PI anchor stop codon Not1); Finally, 190 sol DAF cDNA was constructed into the blunt-ended EcoR1 site of pGEX-5T.

Expression of the gp 190 sol DAF

Strain XL1 containing the human soluble LIF receptor fusion expression plasmid of interest was grown overnight at 30°C in LB medium containing 150 µg/ml ampicillin, diluted 10-fold with fresh LB medium with ampicillin and incubated until the culture reached a A600 of 0.5. Fusion protein synthesis was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (GIBCO BRL) to the culture medium and was allowed to incubate for another 3–4 h at the same temperature. Aliquots of 100 ul were sedimented. The cell pellets obtained were resuspended in 10 µl SDS sample buffer and resolved electrophoretically in an 8% SDS-polyacrylamide gel (SDS-PAGE) under reducing conditions. As a control, strain XL1 was transformed with pGEX-5T. The pGEX-5T directed the synthesis of the 26 kDa glutathione-S-transferase only.

Preparation of gp 190 sol DAF inclusion bodies

The cell culture was spun down and resuspended in 2 ml Tris buffer pH 8.0 (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl) containing 1.0 mg/ml lysozyme (Sigma Chemical, St. Louis, MO) and 1 mM phenylmethysulfonyl fluoride (PMSF) (Sigma Chemical) for 20 min at 0°C and followed by treatment with 1% Triton X-100 for 10 min at 0°C for cell lysis. After sonication (2 × 20 sec), the cell homogenate was separated by centrifugation 15000 rpm for 5 min at 4°C. The pellet containing the insoluble recombinant protein was resuspended in 2 ml 0.1 M glycine-NaOH pH 9.0 with 5 M urea prior to centrifugation in the same condition, then repeating 2 times.

Denaturation of gp 190 sol DAF

The pellet washed by 5 M urea was dissolved in 2 ml 0.1 M glycine-NaOH pH 9.0 containing 8 M urea for 10 min at room temperature prior to sonication (2 × 20 sec). After centrifugation (15000 rpm) for 10 min at 4°C, the supernatant with gp 190 sol DAF was stored at 4°C for further analysis.

Renaturation of gp 190 sol DAF

The lysate in 8 M urea was fractionated by column chromatography using Sephadex G-25 (Pharmacia LKB Biotechnology) equilibrated in renaturation buffer pH 9.0 (50 mM Tris-HCl, 1 mM DL-dithiothreitol (DL-DTT), 0.5 M NaCl, 0.5% Triton X-100, 1 mM PMSF). The fractions containing gp 190 sol DAF were collected and stored overnight at 4°C for the refolding.

Detection of refolding gp 190 sol DAF

Following the refolding reaction, the precipitate was sedimented by the centrifugation and the soluble fraction was filtered through a 0.45 µm filter, and the remaining soluble protein was determined by the absorbance at 280 *nm* (Kontron, Uvikon 930). Meanwhile, the soluble proteins were analyzed by 8% SDS-PAGE under reducing conditions followed by Coomassie blue staining.

Results

Construction of the expression fusion vector pGEX-5T190 sol DAF

A 2.7 kbp cDNA fragment for soluble LIF receptor was cloned into the EcoR1 site (position 965) of the vector pGEX-

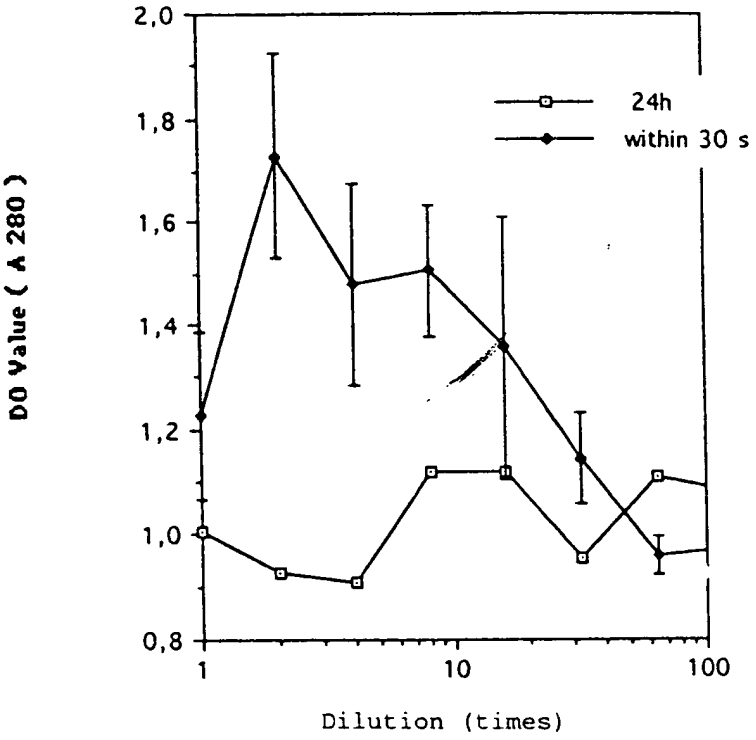

Fig. 1. Concentration dependence of soluble gp 190 sol DAF. After fractionation using Sephadex G-25 to remove urea, gp 190 sol DAF was refolded in renaturation buffer pH 9.0 (50 mM Tris HCl, 1 mM DL-DTT, 0.5 M NaCl, 0.5% Triton X-100, 1 mM PMSF) and diluted between 2- and 100-fold within 30 sec (-◆-) or after 24 h (-□-) at 4°C prior to sedimentation of the precipitate and filtration. The soluble protein was determined by spectrophotometer (Kontron, Uvikon 930) at 280 *nm*.

5T coding for 1117 amino acid (127013 Dalton) of the human soluble LIF receptor. The correct insertion of the cDNA was checked by digestion with multiple restriction enzymes and analysis of the cleavage reactions by gel electrophoresis. The GST-Soluble LIF receptor fusion protein consists of the GST and human soluble LIF receptor with apparent molecular weights of about 26 kDa and 127 kDa respectively.

Concentration dependent of soluble gp 190 sol DAF

Since aggregation requires the encountered of at least two polypeptides, it may be enhanced at high protein concentration. Aggregation might be suppressed by lowering protein concentration in refolding reactions. To examine the influence of protein concentration during folding of gp 190 sol DAF, the lysate without urea fractionated by Sephadex G-25 (about 5 mg/ml) was rapidly (within 30 sec) or slowly (after 24 h) diluted (between 2- and 100-fold) and mixed with renaturation buffer pH 9.0. After the incubation for 24 h at 4°C, the soluble protein was separated by centrifugation and assayed by spectrophotometer at 280 *nm*. The results are presented in Fig. 1. When the protein was diluted from 2- to 10-fold, the refolding gp 190 sol DAF increased about of 40% in the group of immediate dilution (< 30 sec) but not in the group of dilution after 24 h. This demonstrates that when lowering concentration of the denatured protein, the yield of the refolding gp 190 sol DAF can be considerably increased.

Concentration influence of the denaturant on the refolding of gp 190 sol DAF

In our experiment, the concentration of urea was first 5 M to wash the inclusion bodies of gp 190 sol DAF; then 8 M for solubilization of the fusion protein; finally the difference (6–0.05 M) for aggregation and renaturation of the denatured protein by adjustment with renaturation buffer pH 9.0. After the incubation for 24 h at 4°C, the refolding protein was separated and assayed. From Fig. 2, we found that the refolding protein increased constantly following the decrease of urea, meanwhile, was not affected in presence of a little urea.

Slow refolding of gp 190 sol DAF

Kinetic complexities are encountered almost universally in protein refolding. These complexities usually result from conformational heterogeneity of the refolding states with slow- and with fast-refolding molecules [6]. The 190 sol DAF

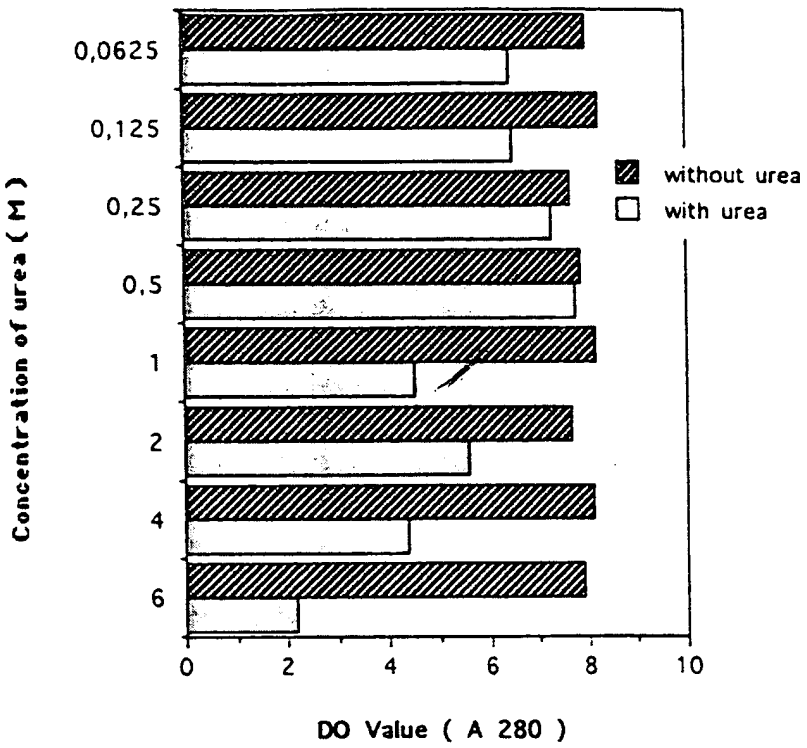

Fig. 2. Concentration influence of the denaturant on the refolding of gp 190 sol DAF. The lysate containing gp 190 sol DAF was separated from urea by Sephadex G-25 and immediately adjusted into various concentrations of urea (□) with the renaturation buffer pH 9.0, and incubated for 24 h at 4°C prior to dialysis with molecularporous membrane (MWCO: 6-800, Spectra/Por. California) against the renaturation buffer pH 9.0 overnight at 4°C to remove urea. Finally, the soluble protein was separated by centrifugation and filtration and was detected by spectrophotometre at 280 *nm* in various concentration aliquots. Each sample was matched a positive control treated similarly but without urea (▨).

molecule was observed about the slow refolding transition. The precipitate of gp 190 sol DAF fusion protein after refolding in renaturation buffer pH 9.0 for 24 h at 4°C was diluted with the same buffer (between 2- and 100-fold), and the renaturation reaction continuously proceeded for 24 h at 4°C. The results showed that although the soluble protein was still presented, the dilution degree did not influence the renaturation reaction of the slow refolding transition (Fig. 3). It suggests that there is a few slow refolding (after 24 h) in the process of the aggregate formation.

Effect of glycerol in refolding of gp 190 sol DAF

As glycerol at 10–20% often helps to maintain stability and is compatible with most purification steps at these concentrations [7], its efficiency was analyzed in our protocol. Sephadex G-25 column was equilibrated with 20% glycerol or none in buffer 0.1 M glycine-NaOH pH 9.0, and the gp 190 sol DAF was eluted by the same buffer, then immediately diluted with one volume (0.5 ml) of the renaturation buffer pH 9.0, with or without 20% glycerol respectively, and finally let overnight at 4°C to refold. From Fig. 4, we observed that the soluble fractions in the group with glycerol were lower than those without glycerol, and eluted easier than the latter. These results suggest that glycerol is disadvantageous to the renaturation of the protein in unfolded formation and advantageous to the elution of protein from the column.

pH affecting the solubilization of gp 190 sol DAF

The aggregates of protein generally resistant to solubilization with native buffers require for their recovery such conditions as extreme pH and high concentration of solute denaturants [6]. Therefore, after urea was removed from the lysate, we found that the solubilization of gp 190 sol DAF was greater in pH 9.0 and pH 4.0 than in pH 7.2 and greatest in pH 9.0. Otherwise, in the 5 M urea group, there was always a lot of soluble protein (Fig. 5).

Discussion

The pGEX vectors were designed for foreign polypeptides to be expressed in XL1 in a form that allows them to be purified rapidly under nondenaturing conditions. Foreign

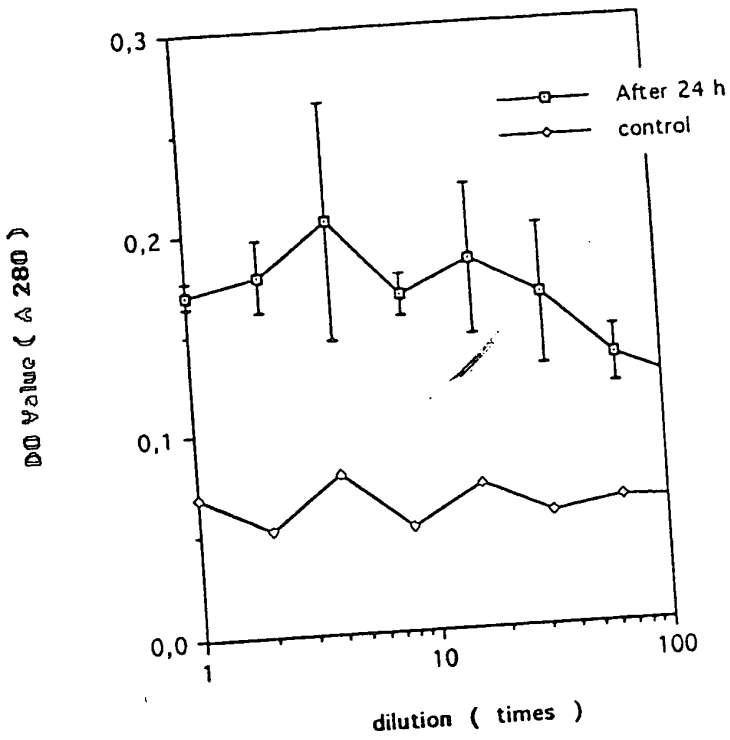

Fig. 3. Slow refolding of gp 190 sol DAF. After refolding in renaturation buffer pH 9.0 for 24 h at 4°C, the precipitate was sedimented by centrifugation 15000 rpm for 10 min at 4°C, then diluted with the same buffer to different degrees (-□-) or not (-◇-) prior to renaturation reaction for 24 h at 4°C. The soluble protein was separated by centrifugation and filtration. Protein was detected by spectrophotometer at 280 nm.

polypeptides are expressed as fusions to the C terminus of GST, a common 26 kDa cytoplasmic protein of eukaryotes. The GST gene used to generate the pGEX vectors was originally cloned from the parasitic helminth schistosoma japonicum [4]. We used the vector pGEX-5T to express the 190 sol DAF fusion protein with an histidine-hexapeptide and the GST at its N-terminus and the recombinant protein at its C-terminus. Therefore, it allows the purification of the 190 sol DAF fusion protein to homogeneity with a single-step affinity chromatography using immobilized glutathione.

However, gp 190 sol DAF is a large molecule about 130 kDa and remains entirely in the insoluble cell fraction [5] as XL1 doesn't play an active role in carrying out essential post-translational modifications folding, and assembly (such as disulfide bond formation, glycosylation and acetylation) [9]. The inclusion bodies are formed in dense intracellular structures and have poor solution properties (aggregation and precipitation). The major problem becomes one of recovering biologically active protein in high yield. In order to accomplish this the protein in the inclusion bodies must be solubilized, refolded, and purified, in a specific order [10].

The inclusion bodies are solubilized with denaturants in a common procedure. When XL1 containing pGEX-5T 190 sol DAF was lysed, gp 190 sol DAF was separated as an insoluble formation and solubilized by 8 M urea. Urea is a potential hydrophobic interaction-disrupting agent focused on its obvious potential for hydrogen bonding. It is considered to act by breaking protein hydrogen bond, and it undoubtedly does interact with peptide groups in unfolded protein by hydrogen bonding. We used urea at various concentrations to process the insoluble ad soluble gp 190 sol DAF before or after the lysis and observed that washing the inclusion bodies of gp 190 sol DAF with up to 5 M urea only solubilized the contaminants (nucleic acid, phospholipids, ad lipopolysaccharides) (Fig. 6), but as the urea concentrations increased to 6 M, the gp 190 sol DAF is partially solubilized as well (data not shown). Otherwise, the folding protein increased constantly following the decrease of urea and was not affected in presence of a little urea (< 0.5 M) in the renaturation reaction of gp 190 sol DAF (Fig. 2). It suggests that the concentration of urea must be kept up to a high enough level for solubilizing gp 190 sol DAF and must be down to a minimum level for refolding gp 190 sol DAF.

Concerning renaturation buffer, ionic strength and pH are obvious variables in the search for conditions that favour productive collapse of an intermediate over its aggregation. First, Tris-HCl was chosen in our renaturation reaction. Then, on condition of certain ionic strength (0.5 M NaCl), reduced dithiothreitol (DL-DTT 1.0 mM) might directly reduce thioredoxin that can catalyze the interchange of these

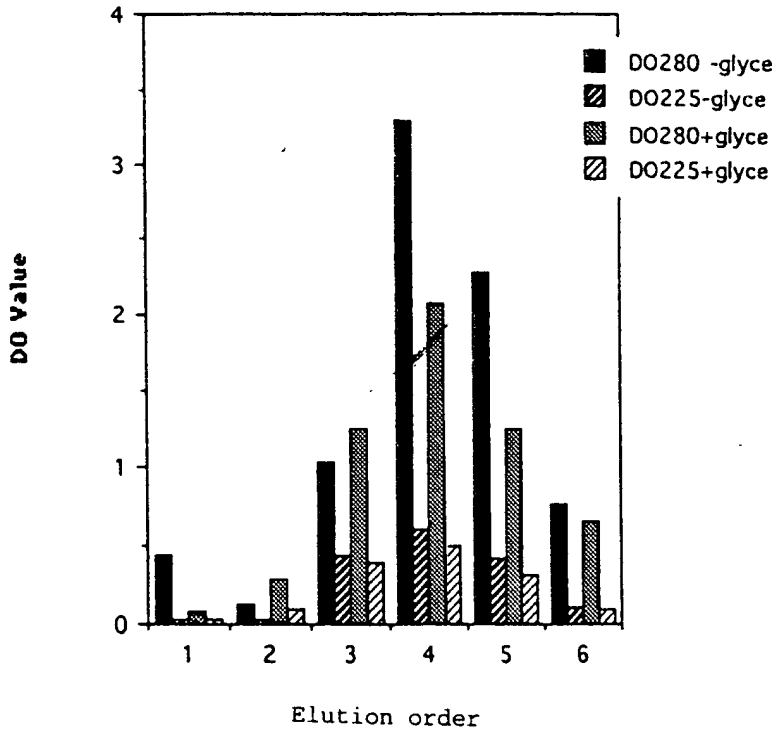

Fig. 4. Effect of glycerol in refolding gp 190 sol DAF. The lysate containing gp 190 sol DAF in 8 M urea is fractionated by column chromatography Sephadex G-25 equilibrated in 0.1 M glycine-NaOH pH 9.0 with (DO 280 □, DO 225 ▨) or without (DO 280 ■, DO 225 ▨) 20% glycerol. The fractions were collected sequentially with 0.5 ml aliquots prior to dilution with the renaturation buffer pH 9.0 with 20% glycerol or not respectively. For refolding overnight at 4°C, the solubilized gp 190 sol DAF was separated by centrifugation (15000 rpm) for 10 min at 4°C and determined by the ultraviolet at 280 *n*m (urea at 225).

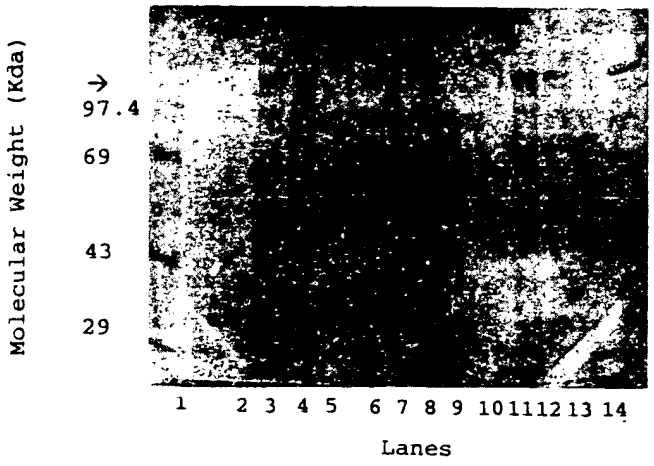

Fig. 5. Solubilization of gp 190 sol DAF at several pHs. The lysates were prepared with 8 M urea in pH 4.0 (lanes 2–5), pH 7.2 (lanes 6–9) and pH 9.0 (lanes 10–13) respectively. Column chromatography Sephadex G-25 (Pharmacia LKB Biotechnology) was equilibrated and eluted at the renaturation buffer at the relevant pH. Meanwhile, the lysate pH 9.0 was added with 5 M urea as a control. After refolding for 24 h at 4°C, the samples were centrifuged for 10000 rpm 5 min at 4°C. The supernatants were migrated and analyzed by 8% SDS-PAGE (lane 1, protein molecular weight standards; lanes 2, 6, 10: 1st elusion; lanes 3, 7, 11: 2nd elusion; lanes 4, 8, 12: 3rd elusion; lanes 5, 9, 13: 4th elusion; lane 14: lysate with 5 M urea).

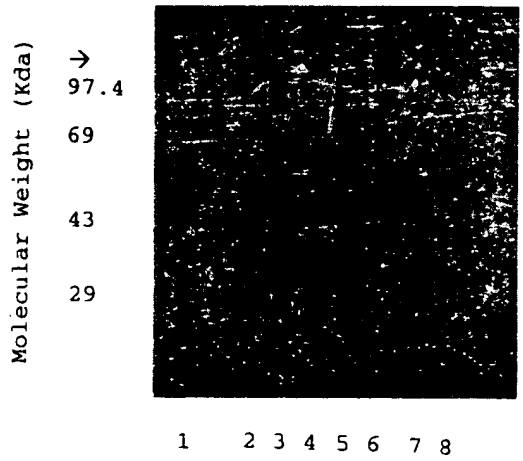

Fig. 6. Washing effect of 5 M urea for 190 sol DAF fusion protein. The inclusion bodies were removed from the lysozyme buffer and resuspended in the liquid (0.1 M glycine-NaOH pH 9.0 lanes 2 and 3) or 5 M urea with 0.1 M glycine-NaOH pH 9.0 (1 x: lanes 4 and 5; 2 x: lanes 6 and 7) prior to incubation for 5 min at room temperature. After centrifugation (15000 rpm) for 5 min at 4°C, aliquots of the pellets resuspended in 8 M urea (lanes 2, 4, 6) and the supernatants (lanes 3, 5, 7) were analyzed by 8% SDS- PAGE. The proteins were stained with Coomassie Blue. Lane 1: molecular weight markers: lane 8: the positive control.

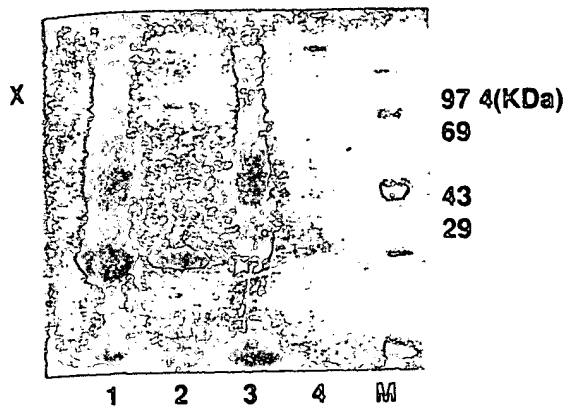

Fig. 7. Purification of 190 sol DAF fusion protein. *Escherichia coli* strain XL1 transformed with pGEX-5T (lanes 1 and 2) and pGEX-5T 190 sol DAF (lanes 3 and 4) was cultured at 30°C induced with 1 mM IPTG for 4 h. After process with lysozyme and sonication, the inclusion bodies were separated and washed (see Materials and Methods) prior to dissolving in 8 M urea. In order to refolding the fusion protein after the denaturation by urea, the fusion protein was fractionated by column chromatography using Sephadex G-25 from urea and allowed in the renaturation buffer pH 9.0 overnight at 4°C, then purified by affinity chromatography using a glutathione-Sepharose 4B column. 100 µl aliquots of the fusion protein before (lanes 1 and 3) and after the purification (lanes 2 and 4) were analyzed with 8% SDS-PAGE gel.

incorrectly paired disulfide [11], ad 0.5% Triton X-100 in the refolding mixture might help to preserve the folded product in solution. The role may be to mask hydrophobic surfaces on folding intermediates transiently, suppressing their aggregation and precipitation, and thus facilitating completion of folding [12]. Otherwise, pH must be considered at the refolding stage, generally be should avoid pHs near the pI of one's proteinin refolding experiments [8]. Though pI of gp 190 sol DAF is yet not clear, our results have also supported this viewpoint, the solubilization of gp 190 sol DAF was greater in pH 9.0 and pH 4.0 than in pH 7.2 and the greatest in pH 9.0 (Fig. 5). The addition of neutral solutes such as glycerol is to stabilize the native states of proteins [13] and can improve *in vitro* refolding yields in some cases [14]. However, we found that glycerol was harmful to the refolding conformation (Fig. 4). Hydrophobic interaction is virtually exclusive to aqueous solution, and glycerol can bring about the interaction decrease whose molecules do not self-associate to the same extent to do H_2O molecules, this ability, essentially paralleling their ability to decrease the surface tension of water or lower its polarity [15]. So, we consider that glycerol in renaturation reaction influences necessary hydrophobic interaction and neutralizes pH's anti-precipitation efficiency. Meanwhile, the precipitation of the fusion protein emerged immediately while pH value of the renaturant buffer (non-urea) was adjusted from 9.0 to 7.2 after the refolding and occurred a little while 20% glycerol was added prior to adjust the pH value in our experiment (data not shown). It is estimated that glycerol might stabilize the refolding state of gp 190 sol DAF.

The concentration of protein in the refolding solution also affects the yield of recoverable active protein. Essentially, large proteins are composed of multiple structural domains, multiple subunits, or both. Although the tridimensional structure of soluble LIF receptor is not known recently, the X-ray crystallography analysis of the structure of the extracellular domain of the human growth hormone receptor shows that each of the 100 amino acid the hematopoietin receptor domain consists of seven strands that form a sandwich of two antiparallel B sheets of four and three strands [16]. These domains apparently can interact with complementary domains of other molecules in the refolding formation, which often leads to aggregation and precipitation of the protein. Therefore, when gp 190 sol DAF unfolding protein was diluted into certain degree, this reaction was decreased and the soluble protein increased. (Fig. 1).

In conclusion, the yield of the refolding gp 190 sol DAF can be considerable increased when lowering concentration of the unfolding protein; a little soluble protein with slow refolding appears in the process of the aggregate formation; The concentration of the denaturant must be down to minimum level for its refolding; ad pH must be considered at its refolding stage.

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